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Sources of *Klebsiella* and *Raoultella* species on dairy farms: Be careful where you walk

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ABSTRACT

Klebsiella spp. are a common cause of mastitis, milk loss, and culling on dairy farms. Control of *Klebsiella* mastitis is largely based on prevention of exposure of the udder to the pathogen. To identify critical control points for mastitis prevention, potential *Klebsiella* sources and transmission cycles in the farm environment were investigated, including oro-fecal transmission, transmission via the indoor environment, and transmission via the outdoor environment. A total of 305 samples was collected from 3 dairy farms in upstate New York in the summer of 2007, and included soil, feed crops, feed, water, rumen content, feces, bedding, and manure from alleyways and holding pens. *Klebsiella* spp. were detected in 100% of rumen samples, 89% of water samples, and approximately 64% of soil, feces, bedding, alleyway, and holding pen samples. Detection of *Klebsiella* spp. in feed crops and feed was less common. Genotypic identification of species using *rpoB* sequence data showed that *Klebsiella pneumoniae* was the most common species in rumen content, feces, and alleyways, whereas *Klebsiella oxytoca*, *Klebsiella variicola*, and *Raoultella planticola* were the most frequent species among isolates from soil and feed crops. Random amplified polymorphic DNA-based strain typing showed heterogeneity of *Klebsiella* spp. in rumen content and feces, with a median of 4 strains per 5 isolates. Observational and bacteriological data support the existence of an oro-fecal transmission cycle, which is primarily

maintained through direct contact with fecal contamination or through ingestion of contaminated drinking water. Fecal shedding of *Klebsiella* spp. contributes to pathogen loads in the environment, including bedding, alleyways, and holding pens. Hygiene of alleyways and holding pens is an important component of *Klebsiella* control on dairy farms.

Key words: *Klebsiella*, environment, bedding, hygiene

INTRODUCTION

Mastitis caused by gram-negative pathogens is a major concern on many well-managed dairy farms in North America. The main gram-negative pathogens that cause mastitis are *Escherichia coli* and *Klebsiella* spp. (Wenz et al., 2001; Roberson et al., 2004; Olde Riekerink et al., 2008). Cows with *Klebsiella* spp. mastitis are more likely to die or to be culled than are cows with other types of mastitis (Erskine et al., 2002; Gröhn et al., 2005; Wilson et al., 2007). *Klebsiella* spp. mastitis causes a considerable and often sustained decrease in milk production, with average losses of 7.6 kg/d shortly after infection and 5 kg/d in subsequent months (Gröhn et al., 2004). *Klebsiella* spp. mastitis responds poorly to antimicrobial treatment (Roberson et al., 2004). Vaccination has been reported to provide some protection against the risk of culling but does not affect the incidence or severity of clinical mastitis due to *Klebsiella* spp. (Wilson et al., 2007). Thus, prevention of exposure is the main strategy for control of *Klebsiella* spp. mastitis.

Sawdust and shavings are important sources of *Klebsiella* spp. (Newman and Kowalski, 1973; National Mastitis Council, 1999). Growth of *Klebsiella* spp. is also observed on mattresses without shavings or other bedding (Kristula et al., 2008). In recent years, *Klebsiella* spp. mastitis has become a problem in herds that use sand bedding. This is attributed to fecal shedding of *Klebsiella* spp. by healthy adult dairy cattle (Munoz et al., 2006). Fecal shedding of *Klebsiella* spp. does not appear to be associated with a chronic carrier state

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Table 1. Characteristics of dairy farms participating in *Klebsiella* spp. study in Northern New York

Item	Farm		
	A	B	C
County	St. Lawrence	Franklin	Clinton
Herd size	1,000	850	270
Production (kg/d)	35.0	37.3	38.6
Bulk milk SCC (cells/mL)	363,500	377,500	290,000
Bedding			
Type	Wood shavings	Sawdust	Sawdust
Replacement	7× per 2 wk	4× per 2 wk	7× per 2 wk
Feeding system	TMR in drive-through bunks	TMR in drive-through bunks	TMR in drive-through bunks
Manure handling	Alley scrapers, set to scrape 2× per h	Alley scrapers set to scrape 1× per h for some pens; skid steer during milking (3×) for other pens	Alley scrapers, set to scrape 2× per h

but rather with frequent uptake and high turnover of a large variety of *Klebsiella* spp. strains that pass through the gastrointestinal tract (Munoz and Zadoks, 2007). In wheat, corn, and alfalfa, *Klebsiella pneumoniae* can act as an endophyte that helps plants to bind nitrogen and bacteria can be found inside the stems and leaves of plants in the absence of external fecal contamination (Chelius and Triplett, 2000; Dong et al., 2003; Iniguez et al., 2004). Oral intake of *Klebsiella* spp. could, therefore, be due to the presence of the organism in or on crops used for feed, or to fecal contamination of feed and water (Figure 1). Fecal shedding of *Klebsiella* spp. resulting in contamination of dairy cattle and their environment is common and it seems likely that exposure of teat ends to *Klebsiella* spp. could result from contact with sources other than bedding (e.g., contaminated alleyways). If such sources could be identified, this might

provide dairy herd managers with additional control points for prevention of *Klebsiella* spp. mastitis.

In our experience as extension veterinarians, most farmers are aware of the role of bedding as a potential source of *Klebsiella* spp. but not of the role of other environmental sources. Scientific studies on the presence of *Klebsiella* spp. in environmental sources other than bedding on dairy farms are rare. The aim of this study was to provide farmers with information on potential *Klebsiella* spp. transmission cycles in the dairy farm ecosystem, considering oro-fecal transmission, transmission via the indoor farm environment, and transmission via the outdoor farm environment.

MATERIALS AND METHODS

Farms

In June, July, August, and September 2007, samples were collected from 3 dairy farms during 1 visit per farm per month. Farms were located in 3 counties in Northern New York. Farm characteristics are summarized in Table 1. Potential for transmission of *Klebsiella* spp. via an oro-fecal cycle was evaluated by sampling of feces, feed, and water. Potential for transmission of *Klebsiella* spp. via the indoor environment was evaluated through sampling of bedding, alleyways, and holding pens. For preliminary assessment of the potential for transmission of *Klebsiella* spp. via the outdoor environment, soil and crop samples were collected.

Sample Collection

Fecal samples ($n = 71$) were collected from the rectum using clean plastic sleeves. Rumen content samples ($n = 19$) were collected from healthy lactating cows with permanently fitted rumen fistulas using clean plastic sleeves. Feed samples included TMR ($n = 12$) from feeding alleys collected after animals had eaten from it

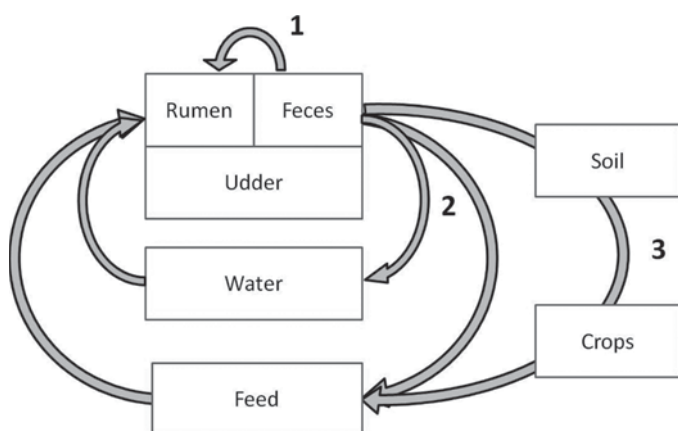


Figure 1. Hypothetical oro-fecal *Klebsiella* spp. transmission on dairy farms showing (1) direct oro-fecal transmission (e.g., through licking of contaminated body parts); (2) indirect oro-fecal transmission via contaminated water or feed without involvement of the external environment; and (3) transmission via contaminated feed with involvement of the environment (soil, feed, and crops).

and TMR or other feed components ($n = 6$) sampled before mixing or feeding. Feed samples were collected in clean zip lock bags of appropriate size. Water samples ($n = 15$) were collected in sterile 240-mL (8 oz) snap cap vials (Capitol Vial, Fultonville, NY). Bedding samples ($n = 57$) were collected from storage piles and from the back 1/3 of stalls (Munoz et al., 2006). Samples from alleyways and holding pens ($n = 53$) consisted of manure slurry picked up from floors using plastic bags or snap cap vials, depending on consistency of the slurry. Soil samples ($n = 42$) were collected from the plough layer of corn and alfalfa plots. Samples from preharvest crops ($n = 19$) were obtained from the same corn and alfalfa plots as were soil samples. In September, only postharvest crops were available and samples ($n = 10$) were collected from corn cut from 10 different fields. Soil and plant samples were stored in clean plastic bags of appropriate size. All samples were stored in cooler boxes with icepacks and transported to the laboratory within 30 h of collection. On-farm activities were conducted with approval from Cornell University's Institutional Animal Care and Use Committee.

Microbiological Analysis

The presence of *Klebsiella* spp. in feces, feed, rumen content, samples from alleyways and holding pens, and soil and postharvest feed crops (corn) was detected using the procedures described by Munoz and coworkers (2006). Briefly, a 1:10 dilution of the sample in saline (1 g of sample in 9 mL of saline) was prepared and incubated for 4 h at 37°C before being streaked onto MacConkey agar containing 10 mg/L of ampicillin (MacA). Preharvest feed crops (i.e., corn and alfalfa plants) were separated into roots, stems, and leaves. Plant parts were surface sterilized for 10 s using 2% of sodium hypochlorite (Clorox Regular-Bleach, The Clorox Company, Oakland, CA) to allow for detection of endophytic *Klebsiella* spp. After bleaching, plant parts were rinsed twice in sterile distilled water before being ground, diluted 1:10 in saline (1 g of sample in 9 mL of saline), and plated onto MacA. For water samples, 150 mL was filtered through 20- μ m Nalgene bottle top filters using a vacuum pump. Filters were taken from bottle tops with sterile tweezers and transferred to a 14-mL culture tube (VWR, West Chester, PA) with 8 mL of Todd-Hewitt broth (CM0189; Oxoid Ltd., Basingstoke, UK). After incubation for 4 h at 37°C, broth was swabbed onto MacA. For bedding samples, 5 g of bedding was mixed with 45 mL of saline using a stomacher. Additional 10-fold dilutions were made in saline and dilutions of 10^{-1} , 10^{-2} , and 10^{-3} were streaked onto MacA. Dilutions of bedding samples were not prepared to determine the bacterial load in bedding but to ob-

tain a bacterial dilution that would allow for selection of isolated colonies from agar for further processing. For all sample types, saline plated onto MacA was used as negative control, and an arbitrarily selected sample inoculated with *K. pneumoniae* ATCC 13883 was used as a positive control for culture procedures. For all sample types, inoculated MacA plates were incubated for 24 to 48 h at 37°C. Putative *Klebsiella* spp. colonies were identified based on colony morphology (Munoz et al., 2006). Based on citrate, motility, and indole testing, isolates were classified as *K. pneumoniae* (citrate positive, motility negative, indole negative) or *Klebsiella oxytoca* (citrate positive, motility negative, indole positive; National Mastitis Council, 1999). A sample was considered positive if a single colony of *Klebsiella* spp. was detected (Munoz et al., 2006).

Molecular Analysis

Previous on-farm studies demonstrated presence of multiple *Klebsiella* and *Raoultella* spp. in bedding, feces, and water but samples from rumen content, soil, and plants were not tested in those studies (Munoz et al., 2006, 2007). Phenotypic methods do not differentiate *Klebsiella variicola* or *Raoultella* spp. from *K. pneumoniae* and *K. oxytoca*. In the current study, genotypic methods (i.e., *rpoB* sequencing; Munoz et al., 2007) were, therefore, used for species identification of isolates from rumen ($n = 73$), soil ($n = 37$), and plants ($n = 27$). In addition to rumen isolates, fecal isolates ($n = 51$) from the same animals were genotypically identified at species level as well as a limited number of alleyway isolates ($n = 15$) from the same farm. Random amplified polymorphic DNA (RAPD) typing (Munoz and Zadoks, 2007) was used to assess and compare strain heterogeneity within paired rumen and fecal samples (i.e., samples obtained from the same cow on the same day). Per sample, 5 isolates were used and RAPD-PCR products from paired rumen and fecal samples were run side by side on agarose gels to allow for direct comparison of banding patterns. Patterns were read by 2 independent observers and any discrepancies in interpretation were resolved as described previously (Munoz et al., 2007).

RESULTS

Feces and Rumen Samples

Of 52 feces samples collected in June, 67% tested positive for *Klebsiella* spp. (Figure 2). Positive samples were detected on each farm. Paired feces and rumen content samples were collected from 12 animals in August and September. Seven animals were sampled twice

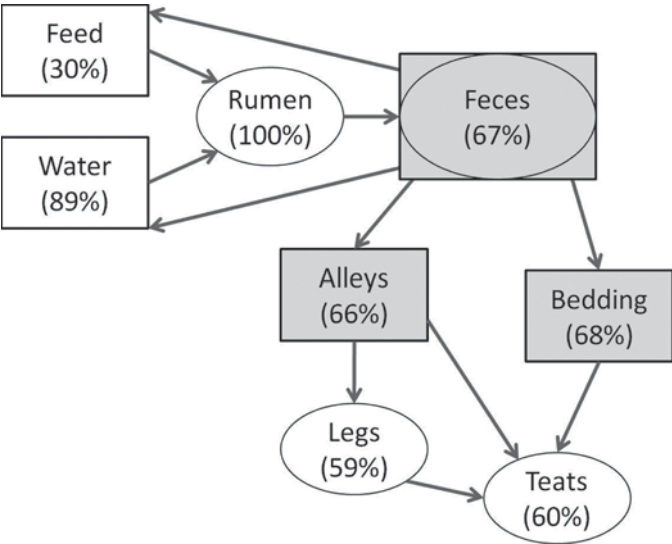


Figure 2. Potential sources and routes of exposure of the mammary gland to *Klebsiella* spp. Ovals represent animals (rectum/feces, rumen, legs, and teats). Rectangles represent environmental sources. Gray rectangles are considered of primary importance for farm management and mastitis prevention. Proportion of samples testing positive based on 3 New York dairy farms described in the current study (feed, water, rumen, feces, alleys, bedding) and Munoz et al. (2008; legs, teats).

and 5 animals were sampled once, yielding a total of 19 sample pairs. All rumen content samples (100%) and 16 of 19 fecal samples (84%) tested positive for *Klebsiella* spp. Based on *rpoB* sequencing, *K. pneumoniae* was the dominant species in both sample types. In addition, *K. variicola* was detected in both sample types. *Klebsiella oxytoca* and *Raoultella planticola* were only detected in rumen content (Table 2). In phenotypic tests, *K. variicola* was identified as *K. pneumoniae*, whereas *R. planticola* could have the appearance of *K. pneumoniae* (indole negative) or *K. oxytoca* (indole positive). For 10 cows, 5 rumen isolates and 5 fecal isolates were available from paired samples. Using RAPD typing, between 3 and 5 strains (median = 4) were detected per rumen sample and between 1 and 5 strains (median = 4) were detected per fecal sample. Within 4 of 10 animals, shar-

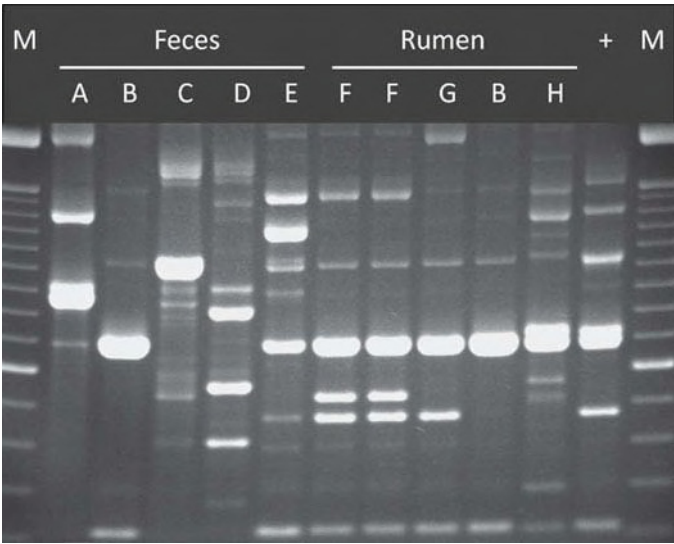


Figure 3. Comparison of *Klebsiella* spp. isolates from feces and rumen content from a single cow on a single day. M = molecular size marker; + = positive control (ATCC 13883); A through H = random amplified polymorphic DNA patterns.

ing of an RAPD pattern between paired rumen and fecal samples was observed (Figure 3).

Feed and Water Samples

Eight of nine water samples (89%; Figure 2) from drinking troughs were positive for *Klebsiella* spp. Positive samples were detected on each farm. Source water used to fill the troughs was tested on one farm, and *Klebsiella* spp. were not detected. Four of twelve TMR samples collected from feeding alleys (30%; Figure 2) and hay (n = 1) and straw (n = 1) collected from storage piles were positive for *Klebsiella* spp. Positive TMR samples were detected on each farm. Haylage (n = 1) and corn silage (n = 3) samples originating from storage piles on a single farm tested negative for *Klebsiella* spp.

Bedding and Alley Samples

Of 53 samples of manure or slurry collected from floors in barns, holding pens, and parlors, 66% tested

Table 2. Distribution of *Klebsiella* and *Raoultella* species (number of isolates; percentage within sample type in parentheses) identified by *rpoB* sequencing among samples from animals, the internal environment, and the external environment of farm C

Source	Samples	Isolates	<i>Klebsiella oxytoca</i>	<i>Klebsiella pneumoniae</i>	<i>Klebsiella variicola</i>	<i>Raoultella planticola</i>
Soil	19	37	13 (35)	4 (11)	2 (5)	18 (49)
Plants ¹	10	27	2 (7)	4 (15)	18 (67)	3 (11)
Rumen	19	73	1 (1)	67 (92)	3 (4)	2 (3)
Feces	16	51	0 (0)	48 (94)	3 (6)	0 (0)
Alleyways	13	15	0 (0)	15 (100)	0 (0)	0 (0)

¹Includes 2 isolates from alfalfa roots (preharvest) and 25 isolates from freshly cut corn originating from 8 corn fields (postharvest).

positive for *Klebsiella* spp. (Figure 2). Positive samples were detected on all farms and originated from alleys within and between pens and parlors, holding pens, and a footbath. Of 57 bedding samples, 68% tested positive for *Klebsiella* spp. (Figure 2). This count includes 5 unused bedding samples of which 2 tested positive for *Klebsiella* spp.

Soil and Plant Samples

Members of the genus *Klebsiella* or *Raoultella* were found in 26 of 42 soil samples. Soil samples originated from the same farm and from a corn field that received annual manure applications (n = 18 samples, 67% positive), an alfalfa field that received annual manure applications (n = 21 samples, 57% positive), and an alfalfa field that had not received manure applications for 50 years (n = 3 samples of which 2 were positive). Per soil sample, multiple isolates were identified to species level using *rpoB* sequencing. *Raoultella planticola* was the most common species in soil samples, followed by *K. oxytoca* (Table 2). Surface sterilized leaves or stems from corn and alfalfa collected preharvest (n = 10 and 9 plants, respectively) did not yield *Klebsiella* or *Raoultella* spp. Among surface sterilized roots from the same plants, 2 alfalfa samples tested positive for *K. oxytoca*. Most postharvest corn samples (8 of 10) showed heavy growth of *Klebsiella* spp., with *K. variicola* as the dominant species (Table 2).

DISCUSSION

This study was designed and conducted in consultation with dairy farmers to address their questions about sources of *Klebsiella* spp. on dairy farms that may contribute to exposure of the mammary gland and to ingestion and fecal excretion of the organisms. Results from this study show that *Klebsiella* spp. are commonly present in environmental sources other than bedding, most importantly in alleyways and holding pens (Figure 2). Presence of *Klebsiella* spp. throughout the dairy farm environment could be expected based on its frequent presence in feces (Munoz et al., 2006, 2007), but participating farmers were not aware of this. We observed that even on farms where managers paid close attention to bedding hygiene and bedding replacement frequency, alley hygiene and maintenance of alley scrapers were neglected. Fecal contamination of the udder, which may be caused by cow traffic via dirty alleyways and holding pens, is associated with increased risk of presence of *Klebsiella* spp. on teat skin before and during milking (Munoz et al., 2008). Anecdotal evidence from farm B collected during the study suggested that improved alley hygiene resulted in lower levels of

teat contamination and lower levels of *Klebsiella* spp. detection after premilking udder preparation. In the current study, levels of *Klebsiella* spp. contamination were quantified as prevalence within sample type. Further quantification of bacterial loads (e.g., cfu counts in samples from different environmental sources), could refine our understanding of the relative importance of bedding, alleys, or other sources in the transmission of *Klebsiella* spp. The simple message, however, is that *Klebsiella* spp. are found wherever manure is found and that bedding management is not sufficient to avoid exposure of teats to these potential mastitis pathogens.

With growing awareness that fecal contamination of the environment is an important source of *Klebsiella* spp., farmers subsequently want to know how *Klebsiella* spp. get into feces, and how fecal shedding of *Klebsiella* spp. might be reduced. Passage of *Klebsiella* spp. from feed to the gut would require survival of the bacteria in the acidic environment of the abomasum, unless the bacteria were protected inside undigested plant matter. Undigested feed materials can reach the gut (Russell et al., 2000) and *Klebsiella* spp. can be present inside feed crops, where they act as endophytes (Chelius and Triplett, 2000). In the current study, based on testing of a limited number of plant samples, endophytic presence of *Klebsiella* spp. inside feed crops could only be detected in roots, parts of the plant that would normally not be eaten by cattle. Protection from gastric acid may also be provided by mixed growth of enterobacteriaceae with acid tolerant bacteria and biofilms (O'May et al., 2005). Survival of *Klebsiella* spp. at low pH may furthermore be enhanced by induction of acid tolerance, a phenomenon that has been described by Aghi and Chhibber (1999). Acid tolerance was induced at pH 5.0 and enhanced bacterial survival at pH 3.5 (Aghi and Chhibber, 1999). One could speculate that low rumen pH, particularly in the case of subclinical rumen acidosis, might, thus, enhance survival of *Klebsiella* spp. during passage from the rumen to the gut. For *E. coli* in cattle and for *Klebsiella* spp. in humans, high starch content in the diet has been claimed to increase fecal shedding of the organism of interest (Russell et al., 2000; Ebringer et al., 2007). Alternatively, or in addition, high rates of passage through the gastrointestinal tract due to high feed volumes may result in decreased killing of bacteria by gastric acid and bile products. Whether increased starch content in bovine rations or increased feed volumes contribute to increased *Klebsiella* spp. levels in the bovine gastrointestinal tract was not investigated. We did demonstrate for the first time, however, that *Klebsiella* spp. were present in the rumen of all cows that were tested, and that multiple species and strains were present in a single gram of rumen content. Both species that were predominantly soil or plant

associated in our study (i.e., *K. variicola* and *R. planticola*; Table 2), and the predominant species from fecal and environmental samples (i.e., *K. pneumoniae*), were present in rumen content. Heterogeneity of *Klebsiella* populations in the gastrointestinal tract is high (Figure 3; Döpfer et al., 2008) and as many as 15 isolates per sample might need to be typed to identify all strains present in that sample with 95% certainty. Against that background, the fact that identical RAPD types were identified in rumen and fecal samples of some cows would appear to suggest that there is flow-through of *Klebsiella* spp. from the rumen to the gut. The flora of farm soil and postharvest crops was dominated by *K. variicola* and *R. planticola*, species that were relatively rare in the rumen. It is unknown whether the rumen does not support the survival or growth of these species to the same extent that it supports survival and growth of *K. pneumoniae*, or whether the rumen is predominantly populated through reingestion of fecal flora, which is also dominated by *K. pneumoniae*. In our opinion, ingestion of *Klebsiella* spp. is most likely due to fecal contamination of feed and water, both because of the prevalence and distribution of bacterial species in different sample types, and because of the likelihood of contact with the various sample types. Further studies would be needed to better understand the levels and relevance of *Klebsiella* species in forage crops and preserved feed and to explore strategies to control levels of ingestion and excretion of *Klebsiella* spp. Such studies could be epidemiological in nature, focusing on identification of herd- or management-level risk factors associated with fecal shedding, or physiological (e.g., focusing on the effect of rations and rumen acidosis on fecal shedding). In addition, the effect of water disinfection and feed storage conditions and preservation methods could be explored. Whether manipulation of ingestion or fecal shedding of *Klebsiella* spp. is technically feasible and economically viable remains to be seen. Meanwhile, knowing that the majority of healthy cows shed *Klebsiella* spp. in feces, farmers should be made aware that bedding is only one of many possible sources of *Klebsiella* spp. and that manure in alleyways and holding pens can contribute to high *Klebsiella* spp. levels on teat skin (Munoz et al., 2008). For practical purposes, hygiene of alleyways and holding pens is of far greater importance than manipulation of dietary ingestion or fecal excretion levels.

In the current study, the genera *Klebsiella* and *Raoultella* were captured under the name *Klebsiella* spp. Although scientifically incorrect, it is common practice in mastitis diagnostics to group pathogens that are phenotypically similar under a single genus name, even if they belong to different genera. Another example would be use of the names *Streptococcus* spp.

or environmental streptococci for species of the genera *Streptococcus*, *Enterococcus*, and *Lactococcus*. *Raoultella planticola*, formerly known as *K. planticola* (Drancourt et al., 2001), was phenotypically indistinguishable from *K. oxytoca* and *K. pneumoniae* and would be classified as a *Klebsiella* in routine diagnostics. Other *Raoultella* species also look like *Klebsiella* spp. on phenotypic testing (Granier et al., 2003; Munoz et al., 2007). In the current study, *R. planticola* was most common in soil and plant samples, consistent with its previously described association with plants, soil, and water (Drancourt et al., 2001). *Raoultella* spp. have also been identified in rumen content (this study) and bedding (Munoz et al., 2007). In humans, *Raoultella* spp. can be of clinical relevance (Granier et al., 2003). To our knowledge, they have not been associated with clinical mastitis or other diseases in cattle but without use of genotypic identification methods, their prevalence and effect may be underestimated.

CONCLUSIONS

Klebsiella spp. are commonly present in soil, water troughs, rumen content, feces, bedding, alleyways, and holding pens on dairy farms. Predominant species in soil and plant samples differ from those in the gastrointestinal tract, water, bedding, alleyways, and holding pens, and the presence of *Klebsiella* spp. in plants appears to result from external contamination rather than endophytic colonization of feed crops. Oro-fecal transmission due to fecal contamination of animals, feed, and water appears more important for maintenance of *Klebsiella* spp. in the farm ecosystem than does introduction from external sources such as fresh bedding or feed. Fecal contamination of stalls, alleyways, and holding pens may result in exposure of teat ends to *Klebsiella*. Further studies would be needed to identify methods to reduce oral ingestion and fecal shedding of *Klebsiella* spp. Meanwhile, hygiene of alleyways and holding pens should be recognized as an important component of prevention of mastitis due to *Klebsiella* spp.

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